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The Transcription Factor *FUSCA3* Controls Developmental Timing in *Arabidopsis* through the Hormones Gibberellin and Abscisic Acid

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Summary

Although plants continually produce different organs throughout their life cycle, little is known about the factors that regulate the timing of a given developmental program. Here we report that the restricted expression of *FUS3* to the epidermis is sufficient to control foliar organ identity in *Arabidopsis* by regulating the synthesis of two hormones, abscisic acid and gibberellin. These hormones in turn regulate the rates of cell cycling during organ formation to determine whether an embryonic or adult leaf will emerge. We also show that *FUS3* expression is influenced by the patterning hormone, auxin, and therefore acts as a nexus of hormone action during embryogenesis. The identification of lipophillic hormones downstream of a heterochronic regulator in *Arabidopsis* has parallels to mechanisms of developmental timing in animals and suggests a common logic for temporal control of developmental programs between these two kingdoms.

Introduction

In higher plants and animals, a molecular appreciation of what controls the timing of specific genetic programs is essential if we are to have a complete understanding of multicellular development. The metamorphosis in insects and amphibians, the onset of puberty in mammals, and the remarkable variation seen in plant morphology often simply represent the consequences of changes in the timing of specific developmental programs relative to one another. In animals, a mechanistic understanding of what determines the timing of developmental programs has mostly been addressed by screening for mutations that advance or retard specific cell lineages during *C. elegans* larval development (see Slack and Ruvkun, 1997, for review). In higher plants, mutational analysis using *Arabidopsis thaliana* has also identified genes that regulate the timing of juvenile and adult leaf transitions and those involved in the conversion from a vegetative to a reproductive mode of development (Poethig, 2003; Bastow and Dean, 2003). To date, however, none of the genes identified through *Arabidopsis* genetic screens appear to be conserved in nematodes, which is perhaps not surprising since plants and animals are thought to have evolved multicellularity independently (Meyerowitz, 2002).

In contrast to the lack of genetic conservation of developmental timing, there appears to be a linkage between terpenoid hormones and heterochrony. In *Arabidopsis*, the terpenoid-based hormone, gibberellin (GA), can influence the time to flowering by stimulating the transcription of the *LEAFY* gene, and in many insects, the terpenoid, juvenile hormone (JH) can affect developmental transitions (Blazquez et al., 1998; Thummel, 2001; Wheeler and Nijhout, 2003). The commonality of a relationship between terpenoid-based hormones and developmental timing might suggest that genetic dissection of newly defined heterochronic pathways will eventually uncover a hormonal component.

In *Arabidopsis*, the ability to easily distinguish cotyledons (embryonic leaves) from vegetative (postembryonic) leaves has allowed the identification of mutations that result in either the replacement of cotyledons with organs more similar to vegetative leaves or vice versa (Baumlein et al., 1994; Keith et al., 1994; Meinke et al., 1994; West et al., 1994; Conway and Poethig, 1997). These mutations can be formally considered heterochronic since cotyledons temporally precede vegetative leaves in the plant life cycle. For example, loss-of-function mutations in the *LEC* genes, *LEAFY COTYLEDON1* (*LEC1*), *LEAFY COTYLEDON2* (*LEC2*), and *FUSCA3* (*FUS3*), result in the replacement of cotyledons with vegetative leaves. A heterochronic interpretation would suggest that the embryo has omitted or advanced the differentiation of the cotyledons, thereby causing these organs to take on a vegetative leaf fate (Keith et al., 1994). Consistent with this, genes that encode markers of late embryogenesis such as seed storage proteins and desiccation protectants are reduced or missing in *lec1* and *fus3* mutants, while germination markers, which normally proceed late embryogenesis, are precociously activated (West et al., 1994; Nambara et al., 2000; Kroj et al., 2003).

Molecular characterization of the *LEC* genes indicates that they regulate cotyledon cell fate by controlling transcription. The *LEC1* gene encodes a protein related to a transcription factor subunit of the *HAP3* gene family in mammals, while *LEC2* and *FUS3* encode proteins of the plant-specific B3 transcription factor *ABI3/VP1* family (Lotan et al., 1998; Luerssen et al., 1998; Stone et al., 2001). Upstream of the *LEC* genes, it has been shown that the CHD3-chromatin-remodeling factor, *PICKLE* (*PKL*), is necessary for repression of these genes outside of the embryo (Ogas et al., 1999; Rider et al., 2003). In turn, *FUS3* appears to negatively regulate the cellular morphogenesis regulator *TRANSPARENT*

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TESTA GLABRA1 (*TTG1*) (Tsuchiya et al., 2004). *TTG1* is thought to be important in the cell specification of many epidermally derived adult structures in the root, hypocotyl, leaf, and seed coat (Koornneef, 1981; Galway et al., 1994; Hung et al., 1998; Western et al., 2001).

Although mutations in the *LEC* genes are developmentally easy to distinguish in the embryo, the difficulty of experimentally manipulating *Arabidopsis* embryos has hindered further understanding of how these genes regulate timing events. In the case of *FUS3*, we have circumvented this problem by expressing it in vegetative leaves. Limiting expression of *FUS3* to the epidermal layer of a vegetative leaf is sufficient to direct all cells within the organ toward cotyledon development, which suggests that a downstream component of this transcription factor acts non-cell-autonomously. Using this tethered system, we found that many *FUS3* misexpression phenotypes are contingent on levels of the two terpenoid-derived hormones abscisic acid (ABA) and GA. *FUS3* regulates where and when ABA and GA are synthesized, and these two hormones in turn determine the stability of the FUS3 protein. Finally, *FUS3* contributes to developmental transitions of leaf identity by regulating the rates of cell cycling, which are also hormonally controlled. Thus, that the heterochronic gene, *FUS3*, specifies the proper schedule of the cycling of cells during cotyledon patterning through terpenoid hormonal signaling is parallel to the logic of developmental timing regulation in other systems.

Results

FUS3 Patterns of Expression Are Influenced by Auxin

We investigated the localization patterns of FUS3 protein during embryogenesis using a reporter *FUS3-GFP* translational fusion under the control of the endogenous *FUS3* promoter (*FUS3::FUS3-GFP*). This translational fusion was fully functional in that it rescued all loss-of-function *fus3* embryonic phenotypes (data not shown). Up to the triangular stage of embryo development, fluorescence was detected in most cells but was more strongly visible in the apical part of the embryo and the suspensor (Figure 1A). By the late heart and torpedo stages, the GFP signal became predominantly confined to the epidermis (Figures 1B and 1C). At these later stages, GFP fluorescence was most pronounced at the cotyledon and root tips with some signal being detected in the vascular tissue within the embryonic root (Figures 1C and 1E). By the walking stick stage, the GFP signal clearly marked the root tip and the epidermal tissues of the cotyledon (Figures 1D and 1F). Closer inspection of the root tip of walking stick embryos revealed weak fluorescence in inner cell layers adjacent to the epidermis, while the cotyledons still showed epidermal-specific fluorescence (Figures 1F and 1G). After this stage, the GFP signal began to dissipate and was completely absent by the time the embryo reached maturity (Figure 1H). These GFP fluorescence patterns support the previously published *FUS3* transcription patterns (Tsuchiya et al., 2004).

The embryonic patterns of FUS3 accumulation are similar to those reported for the auxin-responsive reporter, *DR5* (Friml et al., 2003, Benkova et al., 2003), which suggests that the embryonic patterns of *FUS3* transcript accumulation may reflect differential auxin concentrations. This was tested by dissecting embryos from a *FUS3::FUS3-GFP* transgenic plant and placing the embryos on 100 μM IAA, a naturally occurring auxin. By 24 hr, IAA imbibed embryos showed an increase in the nuclear localization of GFP signal in the root cap cells versus untreated controls (Figures 1I–1L). Because the GFP signal can be induced by exogenous auxin in dissected embryos, we tested the possibility of inducing *FUS3* expression outside of the embryo in an auxin-dependent manner by monitoring the expression of the *FUS3* gene using a *FUS3::GUS* transcriptional reporter. In the absence of exogenous auxin, the *FUS3::GUS* transgenic line normally shows GUS activity in its roots for a duration of approximately 3 days after germination (Figure 1M). After this time, the blue staining dissipated and was only marginally present by 6 days (Figure 1N). After 6-day-old seedlings grown on minimal media were transferred to media containing 10 μM IAA for a 24 hr period, strong GUS activity was observed in the root tip versus roots grown on minimal media (Figures 2N and 2O). A similar incubation on media containing ABA or GA showed little or no increase in blue staining, indicating that the increased GUS activity is auxin specific (Figures 2P and 2Q). Hence, the patterns of *FUS3-GFP* protein accumulation in the embryo may reflect the sites of the auxin maxima, such as the root and cotyledon tips.

Epidermally Derived *FUS3* Is Sufficient to Direct Cotyledon Development

Because the *AtML1* promoter restricts expression to the L1 layer of the shoot apical meristem throughout vegetative and floral development, it allows the misexpression of *FUS3* to be maintained postembryonically (Lu et al., 1996; Sessions et al., 1999). Homozygous *AtML1::FUS3*-GFP seedlings developed into dark green, dwarfed plants that were often semisterile (Figure 2A). After germination, homozygous lines produce foliar leaves that are more similar to cotyledons (Figures 2C–2E). As lateral organs emerge from the meristem, they are yellow, but as they develop take on a rounder shape and a glossy, glabrous surface reminiscent of a cotyledon (Figures 2D and 2E). In strong lines, the production of cotyledons occurred throughout development even after the plant has switched to a reproductive meristem. For example, cotyledonary-like organs appeared from the floral meristem in the same phyllotactic arrangement as that observed for flower organs (Figures 2F and 2G). As the plants age, however, the organs that initially emerged as cotyledonary-like leaves slowly take on features that are more characteristic of vegetative leaves, such as an oblong leaf shape and elongated petioles resulting in a Christmas tree-like appearance (Figure 2B).

As young vegetative leaves emerge from the meristem of misexpressed *FUS3* lines, they accumulate seed storage proteins throughout the leaf, which is consistent with their embryonic identity (Figures 2H–2L). This accumulation is surprising since *AtML1::FUS3-GFP* transgenic plants only show GFP fluorescence in the epidermal layer (data not shown). This result suggests that a downstream component of *FUS3* that regulates seed storage accumulation most likely functions in a cell-nonautonomous manner.

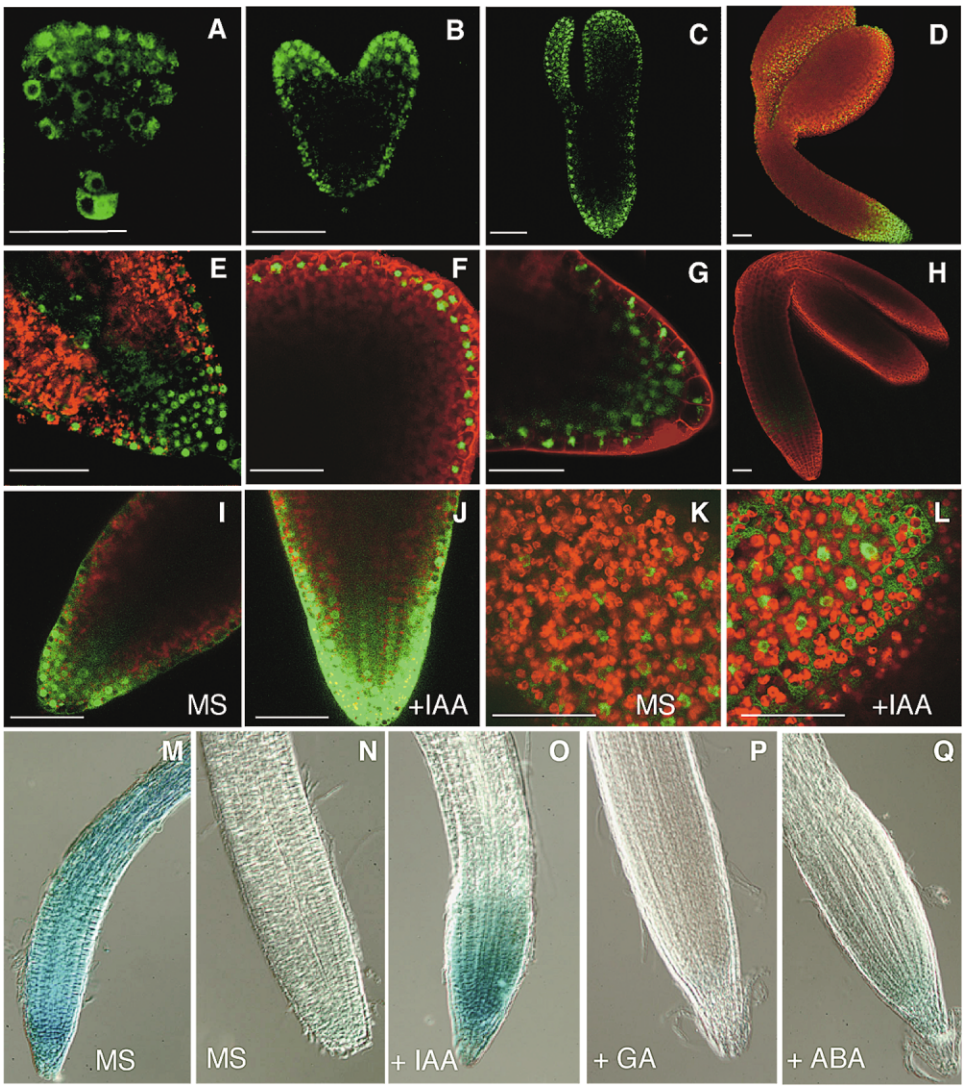


Figure 1. Patterns of FUS3 Expression in the Embryo and Its Regulation by Hormones in the Root

(A–H) GFP localization patterns in *FUS3::FUS3-GFP* embryos at different developmental stages. (A–D) Median longitudinal optical section from triangular (A), late-heart (B), torpedo (C), and walking stick (D) stage embryos. (E) Higher magnification of the root tip at the torpedo stage shown in (C) showing FUS3-GFP localization in the root meristem and provasculature. (F and G) Higher magnification of the cotyledon and root tip from the embryo shown in (D) showing FUS3-GFP expression in the epidermis of the cotyledon (F) and in the root meristem (G). (H) Median longitudinal optical section from a mature stage embryo showing no GFP fluorescence. Propidium iodide staining was performed on embryos at the walking stick (D, F, and G) and mature (H) stages to visualize cell boundaries. The red background in the other images is due to autofluorescence.

(I–L) GFP fluorescence in *FUS3::FUS3-GFP* roots of a walking stick embryo. Ovules were excised from siliques and cultured in the absence (I and K) or presence (J and L) of 100 μM IAA for 24 hr. Images were taken with a constant set of microscopic and image intensity parameters. (I and J) Median longitudinal optical section of root tips. (K and L) Surface view of the epidermis of the hypocotyl region.

(M–Q) *FUS3::GUS* expression pattern in the root tip of 3- (E) or 6- (F–I) day-old seedlings grown on MS media and transferred to MS media (M and N) or MS media supplemented with 10 μM IAA (O), 10 μM GA (P), or 10 μM ABA (Q) for 24 hr.

Bars, 40 μm for (A), (E), (F), (G), (I)–(L); 50 μm for (B), (C), (D), and (H).

FUS3 Positively Regulates ABA Synthesis

ABA has been implicated as a positive regulator of many *FUS3*-regulated embryonic functions including storage reserve accumulation, desiccation tolerance, and dormancy establishment (Keith et al., 1994; Baumlein et al., 1994; Leung and Giraudat, 1998). To test the relationship between *FUS3* and ABA, an ABA auxotrophic mutation (*aba2-2*) was genetically introduced into a *fus3* loss-of-function line that has been complemented with *AtML1::FUS3-GFP* transgene. Because severe *ATML1::FUS3-GFP* lines do not produce functional flowers for crossing, a phenotypically weak *AtML1::FUS3-GFP* transgenic line was used in this experiment. Cotyledon-like leaves produced in weaker lines are mostly lacking in trichome hairs but occasionally produce trichomes at the tip or margins of the leaves (Figure 3A). Flower development is affected to some extent in weak lines, in that the sepals open much earlier than wild-type and growth

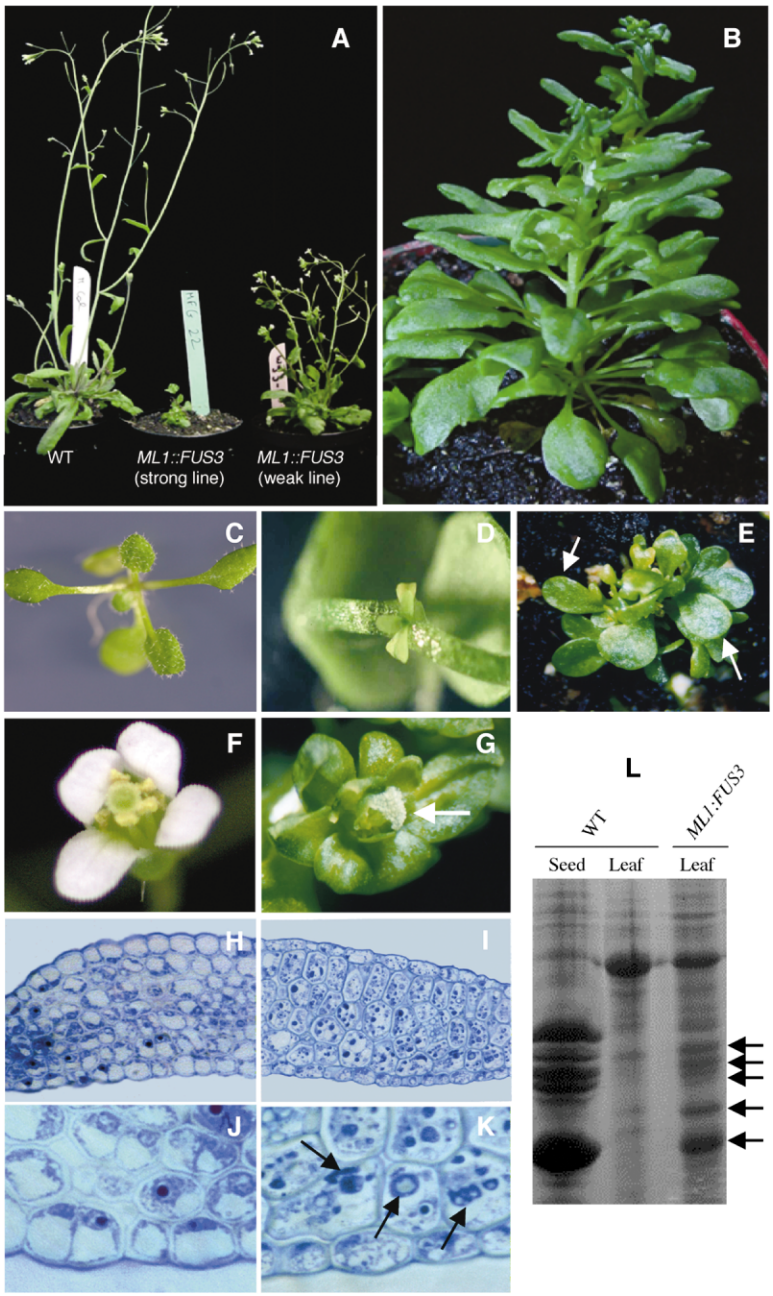


Figure 2. *ML1::FUS3* Vegetative Phenotypes

(A–G) *FUS3* misexpression (*ML1::FUS3-GFP*) in the L1 layer of the meristem strongly reduces plant stature (A) and produces cotyledon-like foliar organs (B–G). (A) 4-week-old wild-type (left), strong *ML1::FUS3-GFP* (middle), and weak *ML1::FUS3-GFP* (right) plants. (B) An 8-week-old *ML1::FUS3-GFP* plant (strong line). (C) A 2-week-old wild-type seedling. (D) A 2-week-old *ML1::FUS3-GFP* seedling (strong line). (E) A 5-week-old *ML1::FUS3-GFP* seedling (strong line). The arrows indicate cotyledon-like leaves. (F) A wild-type flower. (G) A *ML1::FUS3-GFP* flower (strong line). Note the conversion of petals into leaf-like structures. The arrow indicates a carpel.

(H–K) Cross-sections of wild-type leaves (H and J) and *ML1::FUS3-GFP* leaves of a strong line (I and K) stained with toluidine blue. Note that wild-type cells are largely vacuolated as opposed to *ML1::FUS3-GFP* cells, which are densely filled with protein bodies (see arrows).

(L) SDS-PAGE of proteins isolated from wild-type seed (lane 1) and leaves (lane 2), and *ML1::FUS3-GFP* leaves (lane 3). The arrows indicate seed storage proteins accumulating at higher levels in *ML1::FUS3-GFP* leaves compared to wild-type leaves.

of the petals and the filament of the stamens is delayed compared to the growth of the carpel (Figure 3B). These flower defects often result in reduced fertilization, which consequently produces a large proportion of short siliques (Figure 3C). When the *aba2-2* mutation was introduced into the *FUS3* misexpressing line, trichome production was restored on rosette and cauline leaves (Figure 3D), floral defects were rescued (Figure 3E), and plants produced normal elongated siliques (Figure 3F). These results indicate that ABA is necessary for *FUS3* function and that this hormone works at or downstream of *FUS3*.

To more clearly determine the relationship between ABA and *FUS3*, we measured the concentrations of ABA throughout embryogenesis in a *fus3* loss-of-function line (Figure 3G). In wild-type seeds, ABA concentrations were relatively low until 6 days after flowering (DAF), at which time levels began to increase and reached a peak 10 DAF. After this time, they started to decrease again. By contrast, ABA levels in the *fus3* seed showed a similar accumulation pattern to wild-type up to 8 DAF but from then on failed to accumulate ABA to the levels observed in wild-type seeds (Figure 3G). Although these results suggest that *FUS3* is a positive regulator of the ABA biosynthesis, to directly address this, we constructed a *FUS3* inducible system in which the glucocorticoid receptor (GR) from mammalian systems was translationally fused to FUS3 (Aoyama and Chua, 1997). A *FUS3::GR* fusion driven by the *AtML1* promoter was transformed into *fus3* plants. In the absence of the synthetic hormone, dexamethasone (DEX), transgenic seedlings were indistinguishable from the *fus3* parent, but

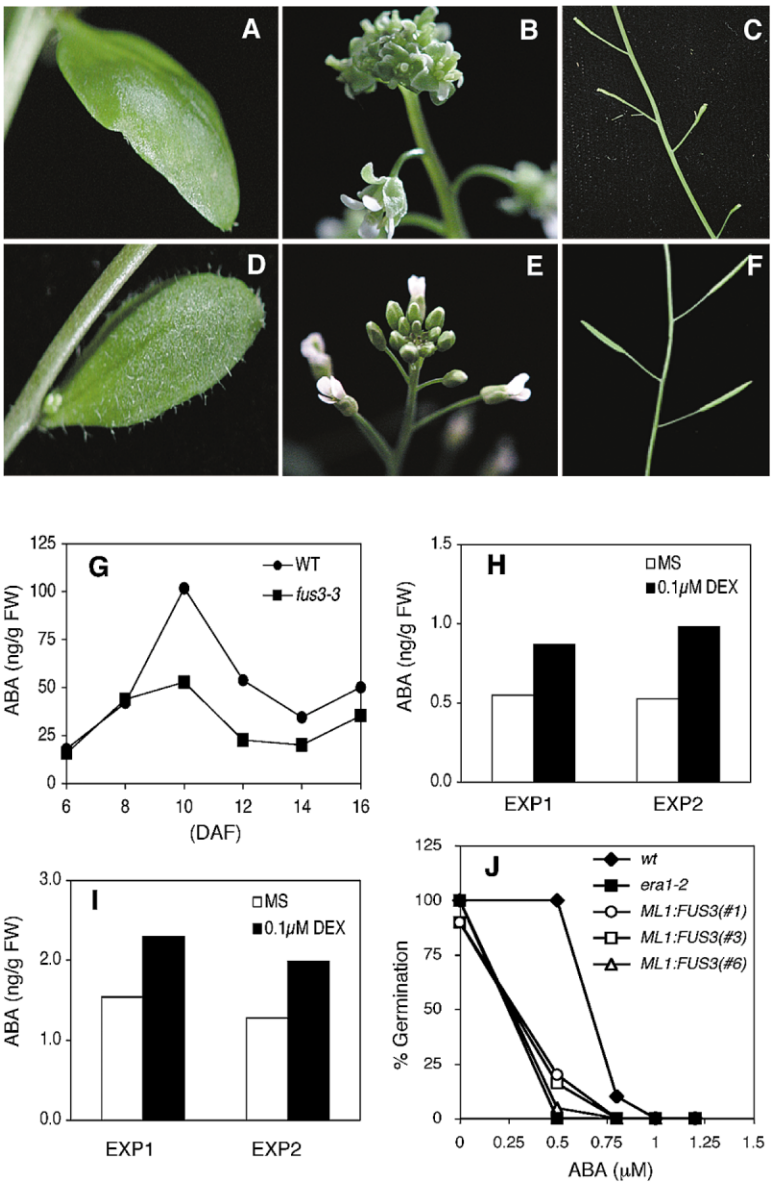


Figure 3. FUS3 Positively Modulates ABA Levels

(A–F) Vegetative phenotypes of *ABA2*, *fus3*, *ML1::FUS3-GFP* (A–C) compared to *aba2-2*, *fus3*, *ML1::FUS3-GFP* (D–F). (A) A glabrous cauline leaf. (B) An inflorescence with open floral organs. (C) A stem bearing short siliques. (D) A cauline leaf bearing trichomes. (E) An inflorescence showing rescued floral organs. (F) A stem with rescued siliques.

(G) ABA levels in wild-type and *fus3* siliques harvested at different days after flowering.

(H and I) ABA levels in *ML1::FUS3-GR* seedlings grown on MS media for 5 days (H) or 9 days (I) and transferred to MS (open bars) or 0.1 μM DEX (filled bars) for 24 hr. Two independent experiments (EXP1 and EXP2) are shown.

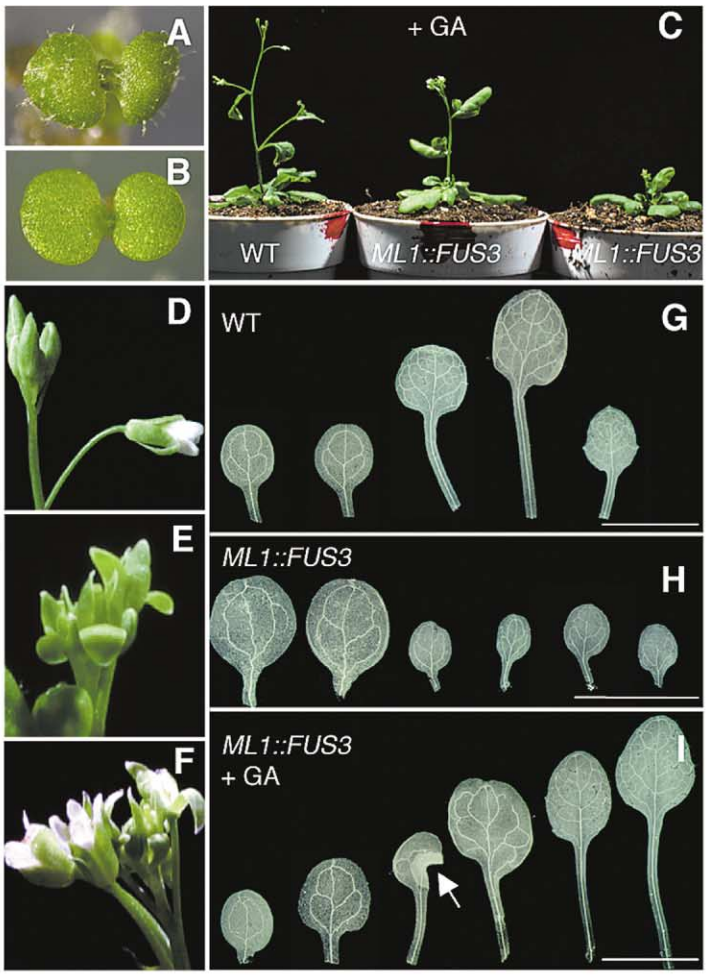
(J) Germination of wild-type, *era1-2*, and three *ML1::FUS3-GFP* lines on exogenous ABA. The sensitivity of *ML1::FUS3-GFP* seeds to ABA is similar to that of the ABA supersensitive mutant *era1-2*. Each point represents a germination test of 50 seeds. The experiment was repeated twice and similar results were obtained.

when germinated and grown in the presence of DEX, transgenic plants produced cotyledonary-like vegetative leaves that are characteristic of *FUS3* misexpression (data not shown). Transfer of 5- and 9-day-old transgenic seedlings from minimal media to low concentrations of DEX for 24 hr immediately increased ABA concentrations in two independent seedling samples (Figures 3H and 3I). Coupled with the observation that loss-of-function *fus3* mutations reduce ABA levels, these results indicate that *FUS3* is a positive regulator of ABA synthesis. The germination of seed from three independent *AtML1::FUS3* transgenic lines all showed increased sensitivity to exogenous ABA versus wild-type, which is consistent with the increased synthesis of ABA in misexpressing lines (Figure 3J).

FUS3 Negatively Regulates GA Synthesis

Many of the adult *FUS3* misexpression phenotypes are reminiscent of a plant defective in GA synthesis or action (Figure 2A; Koornneef and van der Veen, 1980; Steber et al., 1998). Furthermore, GA is important in trichome formation in vegetative *Arabidopsis* leaves and *fus3* loss-of-function mutants have ectopic trichomes on their cotyledons (Keith et al., 1994, Baumlein et al., 1994; Telfer et al., 1997). To explore the role of GA on *FUS3*-dependent functions, a mutation that decreases GA synthesis (*ga1-2*) was introduced into a *fus3* loss-of-function mutant. Double mutants still produced red seeds that were desiccation intolerant, indicating that these *fus3* phenotypes are GA-independent (data not shown). However, ectopic trichome production on cotyledons was reduced and many times absent in double mutants indicating that this phenotype does require GA (Figures 4A and 4B). Consistent with this, when *AtML1::FUS3-GFP* plants were sprayed twice a week with GA, the development was more similar to wild-type plants (Figure 4C). Plants showed a normal stature, bolted on time and both the flower and silique defects were rescued by GA application (Figures 4D–4F).

GA-dependent rescue of *AtML1::FUS3-GFP* lines was



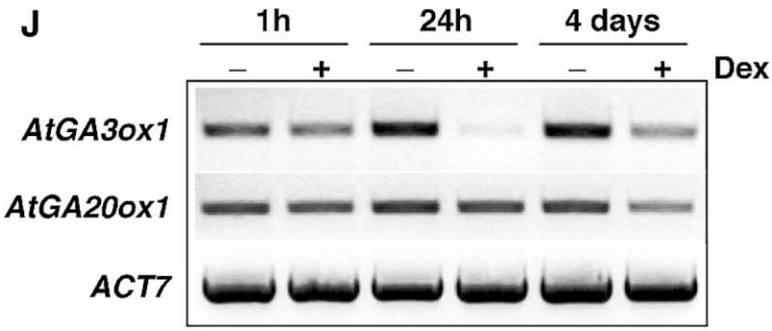


Figure 4. FUS3 Negatively Modulates GA Synthesis

(A) A *fus3*, *GA1* seedling showing cotyledons bearing trichomes.

(B) A *fus3*, *ga1-2* seedling showing glabrous cotyledons resembling wild-type cotyledons.

(C) 22-day-old wild-type (left), *ML1::FUS3-GFP* (right), and *ML1::FUS3-GFP* sprayed with 10 μM GA (middle) showing the rescue of *ML1::FUS3-GFP* late flowering phenotype by GA.

(D–F) Higher magnification of flowers of wild-type (D), *ML1::FUS3-GFP* (E), and *ML1::FUS3-GFP* sprayed with GA (F).

(G) Profile and venation pattern of cleared wild-type cotyledons and vegetative leaves grown on MS media for 4 days and shifted to MS media for an additional 4 days.

(H) Profile and venation pattern of cleared *ML1::FUS3-GFP* cotyledons and vegetative leaves grown on MS media for 4 days and shifted to MS media for an additional 4 days.

(I) Profile and venation pattern of cleared *ML1::FUS3-GFP* cotyledons and vegetative leaves grown on MS for 4 days and shifted to MS supplemented with 10 μM GA for an additional 4 days. The arrow indicates the cotyledon-like leaf sector formed on one half of the leaf.

(J) RT-PCR analysis of *AtGA3ox1* and *AtGA20ox1* expression in the shoot of *ML1::FUS3-GR* seedlings. Tissues were harvested after 1 hr, 24 hr, and 4 days after 1 μM DEX induction (+) compared to control treatment (−). Amplification of *ACT7* is shown as an expression standard.

studied in more detail by following the time of rescue after GA application (Figures 4G–4I). In contrast to an untreated misexpression line (Figure 4H), addition of GA caused a gradual rescue of the leaf phenotypes such as shape, size, venation pattern, and absence of trichomes (Figure 4I). The first vegetative leaf of a seedling grown and germinated on 10 μM GA often resembled an embryonic cotyledon, but occasionally developed as a chimeric leaf showing both embryonic and vegetative venation patterns and shapes (Figure 4I). Indeed, one half of the leaf did not bear trichomes, had a simple venation pattern, and was small in size, thereby resembling embryonic cotyledons (Figure 4I, see arrow), whereas the other half of the leaf was larger, possessed several trichomes, and showed a more complex venation system. Leaf three and successive leaves displayed full rescue of leaf morphology (Figure 4I).

The relationship between *FUS3* and GA is most easily explained by *FUS3* exerting a negative regulation on GA synthesis or action. To test this, the levels of transcripts of two key steps in GA biosynthesis, *AtGA20ox1* and *AtGA3ox1*, were assayed using DEX-inducible *AtML1::FUS3-GR* misexpression lines. Within 1 hr after DEX application, transcript levels of the *AtGA3ox1* showed a slight decrease, and by 24 hr, the signal was highly reduced as measured by RT-PCR (Figure 4J). The

AtGA20ox1 transcript levels also showed a reduction after DEX application, but the kinetics of reduction were much slower as a clear cut decrease in transcript levels only resulted after 4 days on DEX (Figure 4J). These results clearly indicate that *FUS3* is a negative regulator of GA biosynthesis and explain the prevalence of FUS3-dependent GA-related phenotypes.

FUS3 Regulates the Timing of Leaf Development by Controlling Cell Cycling

The ability of GA and ABA levels to modulate *FUS3* misexpression phenotypes is unexpected since these two hormones are not usually associated with specifying organ and cellular morphogenesis. However, both hormones have been suggested to have roles in speeding up or slowing down various aspects of the plant life cycle. Consistent with this, as the *AtML1::FUS3-GFP* lines continued to develop over a longer period of time, the same leaves that started out resembling cotyledons began to develop features that are more characteristic of a vegetative leaf (Figure 2B). For example, before flowering, leaves have a cotyledonary shape and venation pattern, but after having flowered, they have developed a more vegetative venation pattern and leaf shape (Figures 5A and 5B).

The conversion of a cotyledonary leaf into a vegetative leaf over time suggests that the scale of leaf development in a *FUS3* misexpression line is slower than normal. One factor that determines the size, shape, and venation patterns of leaves is the rate of cell divisions during leaf primordial expansion (Kang and Dengler, 2002). To determine if the spatial and temporal patterns of cell cycling in *AtML1::FUS3-GFP* lines are altered, a cyclin β-glucuronidase fusion reporter (*cyc1At::GUS*) was introduced into a *FUS3* misexpression line. This construct has been used extensively to characterize patterns of cell divisions in *Arabidopsis* developing leaves (Donnelly et al., 1999). As expected in wild-type, a gradient of punctate GUS staining, which marks single cell divisions, was detected in young leaves emerging from the apical meristem (Figures 5C and 5D). By contrast, in similarly aged *AtML1::FUS3-GFP* lines, blue staining was highly reduced in emerging foliar organs, suggesting that *FUS3* is a negative regulator of cell cycling in *Arabidopsis* (Figures 5E and 5F).On this note, the ability of GA to rescue *AtML1::FUS3-GFP* phenotypes suggests that application of GA may function to increase the cycling of cells in *FUS3* misexpressing lines. As expected, increased GUS staining was observed when GA was applied to *AtML1::FUS3-GFP* (Figures 5G and 5H). It therefore appears that the GA rescue of *AtML1::FUS3-GFP* phenotypes is due to the ability of this hormone to increase cell cycling (see Supplemental Figure S1 at http://www.developmentalcell.com/cgi/content/full/7/3/373/DC1).

ABA and GA Feed Back to Modulate the Stability of *FUS3* Protein

In the past few years, a number of studies have indicated the role of plant hormones as regulators of the turnover of key proteins involved in hormone signaling (Devoto et al., 2002; Dharmasiri and Estelle, 2002; Dill et al., 2001; Potuschak et al., 2003; Gao and Ecker, 2003). To explore a possible role of ABA and GA on FUS3 protein stability, we monitored the GFP fluorescence in *AtML1::FUS3-GFP* vegetative leaves after exposure to ABA, GA, and an inhibitor of GA biosynthesis, uniconazole-P. In the presence of ABA, GFP fluorescence is stabilized compared to untreated controls, suggesting that the upregulation of ABA biosynthesis by FUS3 may in turn stabilize the *FUS3* protein in vivo (Figures 6A and 6B). In contrast, a 4 day exposure to 10 μM GA slowly decreased the GFP signal versus control samples (Figure 6D). Consistent with this observation, GFP fluorescence was strongly localized to the nuclei in the presence of 10 μM uniconazole (Figure 6C). The ability of ABA to stabilize FUS3 protein explains why decreased ABA concentrations using an ABA-deficient mutant suppressed *AtML1::FUS3-GFP* phenotypes. Similarly, the decreased stability of FUS3 protein in the presence of GA explains why exogenous application of GA suppressed *FUS3* misexpression phenotypes.

Discussion

FUS3 Is a Nexus of Hormone Action in the Embryo

During *Arabidopsis* embryogenesis, the rise in ABA levels is important for the establishment of many late embryogenic functions. Conversely, GA concentrations remain relatively low during this period until mature seeds are imbibed, at which time GA rapidly increases, thereby reversing ABA-induced dormancy (Ogawa et al., 2003). At a molecular level, GA influences a number of key regulators of ABA signaling, which suggests that decreases in ABA concentrations in conjunction with increases in GA levels act together to modulate ABA-dependent gene expression during seed development and germination (Ogawa et al., 2003). A coordination of GA and ABA levels during embryogenesis and germination must therefore be upheld so that the embryo does not receive mixed hormonal messages.

One way that *FUS3* can coordinate the ABA/GA action in the embryo is through feedback loops produced by *FUS3* regulating the levels of ABA and GA and these hormones regulating the stability of FUS3 protein (Figure 7A). The production of *FUS3* in the epidermis and later in the cotyledon margins, root tip, and vasculature of the embryo inhibits production of GA in these tissues (Figure 7A). Thus, GA-dependent processes involved with vegetative leaf development such as trichome production, venation patterns, and cell expansion would be suppressed. This is supported by the observation that *AtGA3ox1*, a gene that encodes the last step of GA biosynthesis, is quickly downregulated by the activation of FUS3 protein (Figure 4J).

During late embryogenesis, the situation would be reversed by GA synthesis (Figure 7B). The site of biosynthesis of active GA in *Arabidopsis* embryos during germination does not coincide with the expression of GA-responsive genes, suggesting that GA or a GA signaling component is cell nonautonomous (Ogawa et al., 2003). Thus, the long-range action of GA synthesized in tissues where FUS3 is not expressed would ensure that epidermally derived FUS3 protein is degraded so that normal development can progress (Figure 7B). Consistent with this, transcript levels of *AtGA3ox1* are low

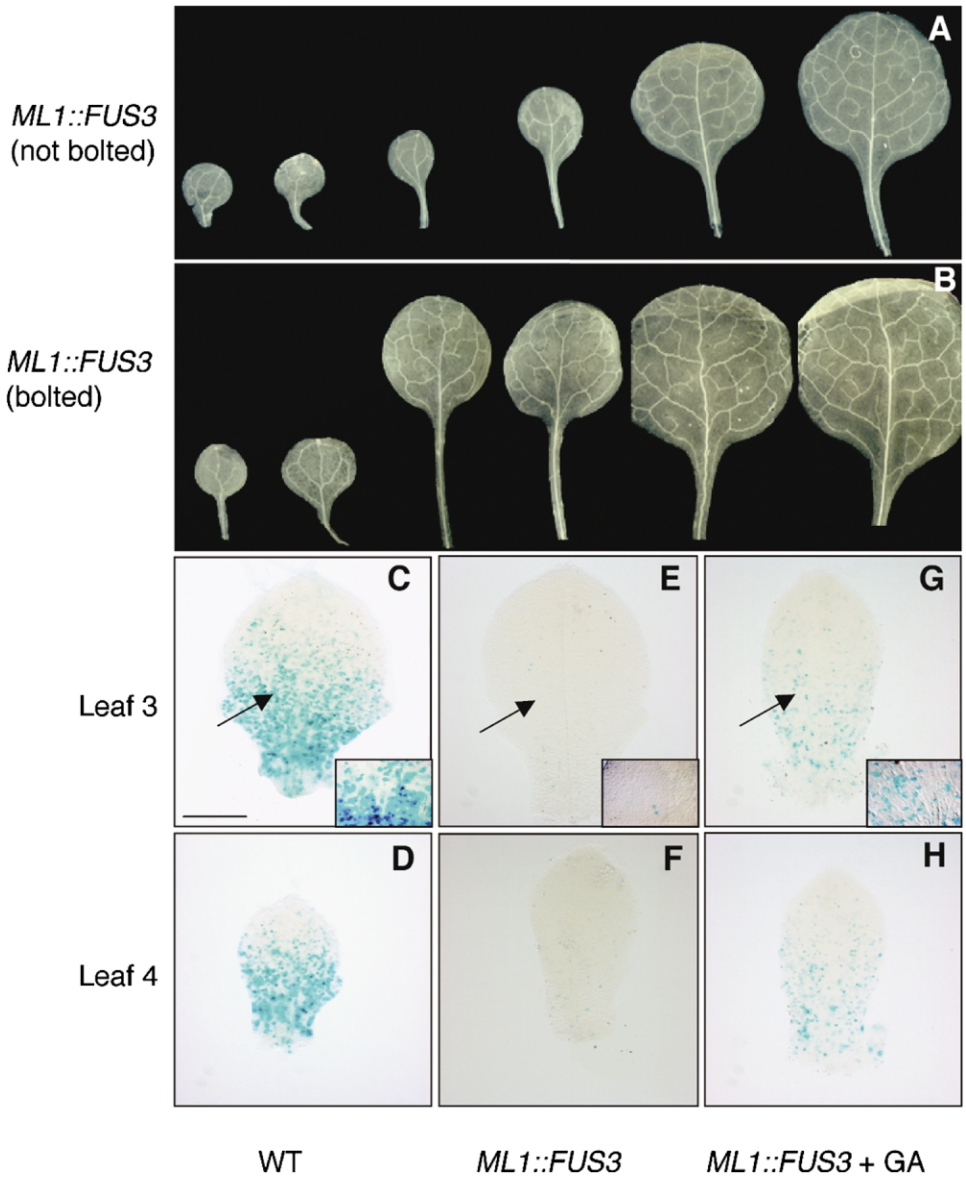


Figure 5. FUS3 Affects Leaf Development by Reducing Cell Cycling

(A and B) Leaf profile of cleared *ML1:FUS3* cotyledons and leaves 1 to 4 grown on soil for 22 days. (A) A 22-day-old *ML1::FUS3* plant, not bolted. Note the venation patterns of leaves 1 and 2 resemble that of the cotyledons. (B) An *ML1::FUS3* plant, bolted. Note that the size and venation patterns of leaves 1 and 2 is partially rescued.

(C–H) *Cyc1At::GUS* expression patterns in cleared whole mounts of 7-day-old wild-type and *ML1::FUS3* leaves. (C and D) Leaf 3 (C) and 4 (D) of wild-type seedlings grown on MS media. (E and F) Leaf 3 (E) and 4 (F) of *ML1::FUS3* seedlings grown on MS media. (G and H) Leaf 3 (G) and 4 (H) of *ML1::FUS3* seedlings grown on MS media supplemented with 10 μM GA.

Insets in (C), (E), and (G) show higher magnification of leaf sectors as indicated by the arrows. Bars, 100 μm for (C)–(H).

Between 8 and 10 seedlings per genotype were stained for GUS activity and leaves 3 and 4 of the same age were photographed. Similar patterns of GUS staining were obtained and the leaves shown are representative for each genotype.

during early stages of embryogenesis in wild-type seeds and only increase later in mature green embryos, a stage that roughly coincides with the disappearance of FUS3 protein in the embryo (Yamaguchi et al., 1998).

The negative regulation of GA concentrations by *FUS3* also sheds some light upon the relationship between this transcription factor and its upstream regulator, *PKL*. Loss-of-function *pkl* mutants grow as dark-green dwarfs, suggesting that this mutant may be defective in GA synthesis or signaling (Henderson et al., 2004). The similarity of *pkl* loss of function and *FUS3* misexpression phenotypes with respect to GA-dependent growth processes suggests that one function of *PKL* is to repress *FUS3* in leaves. Thus, GA is synthesized to allow the normal progression of vegetative leaf development. This relationship is consistent with the recent report showing that *pkl* seedlings have substantially higher levels of *FUS3* transcripts (Rider et al., 2003).

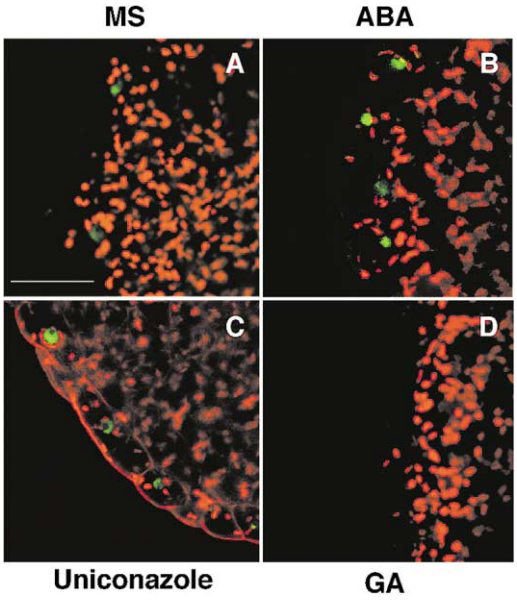


Figure 6. FUS3-GFP Stability Is Affected by Hormones

(A–D) Confocal images of GFP accumulation in the epidermis of *ML1::FUS3-GFP* leaf primordia. *ML1::FUS3-GFP* seedlings were grown on MS media and shifted to MS (A), 10 μM ABA (B), 10 μM uniconazole-P (C), or 10 μM GA (D) for 4 days. Images were taken with a constant set of microscopic and image intensity parameters. Bar, 70 μm for (A)–(D).

Coincident with early *FUS3*-dependent suppression of GA synthesis in the epidermis is the induction of ABA synthesis in this tissue. Epidermally derived ABA appears to have two purposes: to stabilize FUS3 protein which reinforces the negative control of GA synthesis by FUS3, and to diffuse to other tissues where it can influence other late embryogenic processes such as reserve storage accumulation, desiccation tolerance, and the establishment of dormancy (Figure 7A). Although this model would predict that the cell-nonautonomous component of FUS3 action is ABA, this hormone alone is not sufficient to instruct late embryogenic gene expression. *Arabidopsis* wild-type leaves do not accumulate embryonic markers such as seed storage proteins upon exogenous ABA application unless the *ABI3* transcription factor is also ectopically expressed in the adult plant (Parcy et al., 1994). Possibly, *FUS3*-derived ABA acts in combination with *ABI3* to instruct late embryogenesis (Figure 7A).

Finally, because FUS3 transcription is auxin inducible, the pooling of auxin during patterning of the embryo may determine the sites of FUS3 expression during embryogenesis. During the transition from globular to triangular stages of embryogenesis, auxin moves through the epidermal layer of cells and accumulates at the incipient cotyledon tips (Benkova et al., 2003; Friml et al., 2003). Subsequently, auxin is drained through the inner cell layers toward the basal region that will form the future embryonic root (Benkova et al., 2003; Friml et al., 2003). By tying *FUS3* expression to dynamic auxin gradients formed during embryogenesis, auxin may modulate the levels of two other hormones, GA and ABA, in regions of future organogenesis (Figure 7A).

FUS3 and the Control of the Cell Cycle

The ability of epidermally derived FUS3 to coordinate ABA and GA synthesis does not explain how these two hormones contribute to the specification of cotyledon cell fate. In *Arabidopsis*, the embryonic epidermal layer becomes distinguished in young dermatogen embryos, but it is not until the late globular stage that cell divisions in this layer increase in frequency in the regions that will be the future cotyledons (Mansfield and Briarty, 1992). Unlike the shoot apical meristem, misexpression of *FUS3* does not instruct root meristematic cells to take on an embryonic leaf fate, which suggests that *FUS3* plays a permissive role in foliar organ identity. A permissive rather than an instructive role for *FUS3* is consistent with this gene controlling two hormones that appear to regulate the speed of plant growth rather than instructing specific developmental programs. For example, in GA-deficient mutants, the appearance of vegetative traits, like trichomes, is delayed and the expression of many juvenile traits is prolonged (Evans and Poethig, 1995).

Loss-of-function *fus3* mutants show ectopic cell divisions in the embryo, suggesting that one function of *FUS3* involves negatively regulating cell division (Raz et al., 2001). The reduction in cell cycling observed in vegetative leaves misexpressing *FUS3* supports this contention. After the foliar organ has been patterned in wild-type embryos, *FUS3* retards further cell division, which in turn may allow late embryogenic programs to be established. In wild-type embryos, seed storage reserve accumulation only rapidly increases after cell divisions in the cotyledons cease (Mansfield and Briarty 1992; Raz et al., 2001). Thus, the inability of *fus3* embryos to cease cell cycling may be the cause of the late embryogenic phenotypes observed in this mutant.

In plants, this relationship between cell cycle progression and developmental decisions is unclear since the cell cycle can be perturbed quite severely with little effect on overall organ shape (Hemerly et al., 1995). However, distinctive tissue types and cellular morphogenesis do appear to be dependent on rates and timing of cell divisions. For example, within a single tissue system such as vasculature, the size of veins and patterns of venation basically reflect the duration of proliferative divisions (Kang and Dengler, 2002). Interestingly, at early stages of *Arabidopsis* leaf development, the formation of primary and secondary veins resembles the simple archetypal pattern seen in mature cotyledons. It is only later, as the leaf continues to grow, that the full complement of tertiary veins, the hallmark of an adult leaf, forms. Normally, in the early-formed foliar organs, continued cell divisions progress the organ toward a more mature vegetative stage. In this scenario, retardation of cell cycling by *FUS3* misexpression during emergence of vegetative leaves results in a venation pattern more akin to an early-formed leaf. The partial rescue of *FUS3* misexpression by time or GA application simply reflects the occurrence of a sufficient number of cell divisions in order to allow the foliar organ to reach a mature vegetative stage.

Evolutionary Convergence on Terpenoid Control of Developmental Timing

The finding that the *FUS3* gene acts through two terpenoid-based hormones, ABA and GA, to ensure the

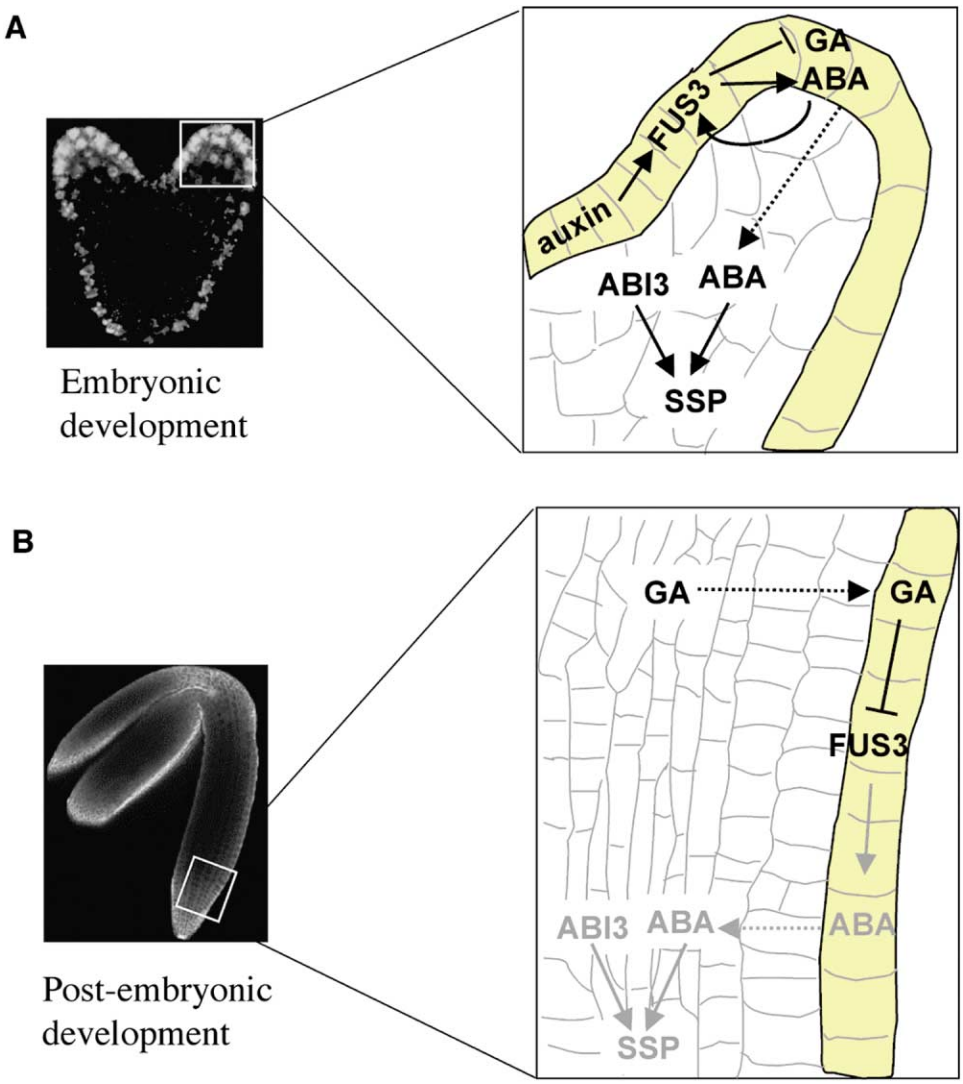


Figure 7. Model of *FUS3* Function during Embryonic and Postembryonic Development

(A) A heart stage embryo showing the role of FUS3 during embryonic development. During embryogenesis, *FUS3* expression follows auxin gradients. Auxin moves through the epidermis (yellow) toward the cotyledon tips upregulating *FUS3* expression in this cell layer. In the epidermis, FUS3 has two roles: it inhibits GA-mediated functions, thus preventing the initiation of a germination program, and it induces ABA synthesis. ABA accumulation in the epidermis stabilizes FUS3 and ABA also diffuses to the mesophyll to induce *ABI3*-mediated accumulation of SSP.

(B) A germinating embryo showing FUS3 function during postembryonic development. Upon imbibition, high levels of GA are synthesized in the embryo and move to the epidermis to inhibit FUS3 functions. This represses the embryonic program dependent on FUS3 and allows the expression of the vegetative program.

Dotted lines indicate hormone movements.

proper temporal progression of leaf development, is intriguing. ABA is chemically very similar to insect JH and animal retinoic acid (Kushiro et al., 2003; Wheeler and Nijhout, 2003). As with ABA in plants, JH slows development in honeybees and increases storage protein synthesis in the presumptive queens (Evans and Wheeler, 1999). Furthermore, JH and ecdysone coordinately control pupal development of the epidermis. Although GA-like molecules have not been discovered in animals, the antagonistic relationship of ABA with GA does share some similarities with the interactions of JH and ecdysone in insects. Parallels have been drawn between larval molting in *C. elegans* and *Drosophila* with respect to the involvement of lipophillic hormone signaling in the control of developmental timing (Thummel, 2001). However, unlike nematodes and flies, which have common evolutionary links, the involvement of terpenoid hormones in developmental timing of cotyledon in *Arabidopsis* could mean convergent evolution has occurred that has permitted these chemical structures to be used for timing of processes in the two kingdoms. It has been speculated that the terpenoid-structure versus other lipophillic hormones may allow these molecules to interact at low affinities with a variety of signaling proteins, thereby allowing coordinated modulation of a range of disparate processes (Wheeler and Nijhout, 2003). Such

a property may explain why terpenoid structures have been used in the evolution of both plant and animal developmental timing. These speculations obviously will require more experiments including the molecular identification of the ABA and JH receptors from both plants and animals.

Experimental Procedures

Plant Material and Growth Conditions

All seeds used in this study were of the Columbia (Col) ecotype. *Arabidopsis* seeds were imbibed for 5 days at 4°C and seedlings were grown at 20°C under constant light on 0.5 X MS plates supplied with the appropriate hormones or inhibitor. Soil-grown plants were also grown under constant light. Dexamethasone (Sigma) was dissolved in DMSO and used in a final concentration of 0.5 μM, while GA_3 (Sigma), ABA (Sigma), and uniconazole-P (Wako) were dissolved in ethanol and used in a final concentration of 10 μM. For external GA_3 application, plants grown on soil were sprayed three times a week with 10 μM GA_3. IAA (Sigma) was dissolved in water and used in a final concentration of 10 μM.

Cloning and Generation of Transgenic Plants

For the construction of the GFP fusions, restriction sites were introduced at the stop and start codon of *FUS3* and *GFP*, respectively, by using oligonucleotide primers and a proofreading polymerase, Vent (NEB Biolabs), for PCR amplification. The resulting PCR product was subcloned into pBluescript, and sequenced. *FUS3-GFP* fragments were cut from pBluescript using KpnI and SacI and then assembled into the pBI101 vector (Stratagene) under the control of the 3 Kb fragment of the *AtML1* promoter (Tsuchiya et al., 2004) to generate *AtML1::FUS3-GFP*, or under the control of the 1.5 Kb fragment of *FUS3* promoter to generate *FUS3::FUS3-GFP*. *AtML1::FUS3-GR* was constructed by replacing GFP in *AtML1::FUS3-GFP* with the glucocorticoid (GR) receptor hormone binding domain (Aoyama and Chua, 1997). These constructs were transformed into *Arabidopsis* by the floral dip procedure (Clough and Bent, 1998) and transgenic T1 plants were selected on 0.5 X MS medium supplemented with 25 μg/ml kanamycin. Primer sequences used to amplify the various PCR fragments were as follows: 5′-AAGGTACCATGAT GGTTGATGAAAATG-3′ (FUS3-KpnI-F); 5′-ATTGAGAATTCCGTAG AAGTCATCGAG-3′ (FUS3-EcorI-R); 5′-TTTCCCGGGCAATGGTGA GCAAGGGCGAGGAG-3′ (EGFP-SmaI-F); 5′-TGGAGCTCCTATCCG GACTTGTACAGCTCGTCCAT-3′ (EGFP-SacI-R); 5′-TAACGTAAGC TTTTCTTCCTCACCCATACTTTCC (FUS3prom-HindIII-F); 5′-CAT GGGTACCTCTCTCAATTGGTTAACACTGCC-3′ (FUS3prom-KpnI-R); 5′-TCTAGAGGATCCGAAGCTCG (GR-F) and 5′-GAACGAGCTCA TTTTTGATGAAACAGAAGC-3′ (GR-SacI-R).

RT-PCR Analysis

RT-PCR analysis of total RNA that had been extracted using Trizol reagent (GIBCO) was performed using One Step RT-PCR Kit (Qiagen). 30 PCR cycles were performed with 250 ng of total RNA to amplify 359 bp of *AtGA20ox1* and 217 bp of *AtGA3ox1*, using primers described in Hay et al. (2002). The amplification of the *ACT7* transcript was used as an expression standard using primers described in Hardtke et al. (2004). Experiments were conducted three times with similar results.

Isolation of *fus3 ga1* Double Mutant

To generate the *fus3 ga1* double mutant, the *ga1-3* mutation from a Lansberg ecotype was introgressed seven times into a Col genetic background and then crossed to the *fus3-3* to obtain F1 population. F2 seed was screened for seeds that were unable to germinate in the absence of 10 μM GA, a common *ga1-2* phenotype. F3 seed from individual *ga1-2* seedlings were grown and screened for the *fus3* purple-red seed color phenotype. A CAPS marker of the *fus3* polymorphism (Tsuchiya et al., 2004) was used to confirm the *fus3* genotype.

fus3 aba2-2 AtML1::FUS3-GFP Mutant Analysis

fus3-3 plants were transformed with *AtML1::FUS3-GFP*, and kanamycin-resistant T1 plants were propagated. T3 *fus3* plants homozygous for the *AtML1::FUS3-GFP* transgene were crossed to *aba2-2* and allowed to self-fertilize. F2 seedlings that were resistant to kanamycin were then transferred to soil. Since *fus3-3 aba2-2* double mutant plants produce black seeds, plants were screened for those segregating 1/4 black seeds in the F3 (Nambara et al., 2000). Because *AtML1::FUS3-GFP* can rescue *fus3* seed color, these F3 plants were expected to be homozygous for *fus3* and *aba2-2* and hemizygous for *AtML1::FUS3-GFP*. In the F4, *fus3*; *aba2-2*; *AtML1::FUS3-GFP* triple homozygous plants, expected to produce only a wild-type seed color, were selected. F4 seedlings homozygous for the *fus3-3* mutation were confirmed by a CAPS marker and the *aba2-2* mutation was detected by diagnostic PCR as follows. ABA2-2F (5′-TGGGCT TACAATTTAAGGCCC-3′) and ABA2-2R (5′-TCTCGTCAAAGTTGT AGTCCTC-3′) primers amplify a 2 Kb fragment of the *ABA2* gene using wild-type genomic DNA as template, but fail to amplify a PCR product using *aba2-2* genomic DNA.

Histochemistry and Microscopy

The *Cyc1At::GUS* and *FUS3::GUS* transgenic lines have been previously described (Donnelly et al., 1999; Tsuchiya et al., 2004). Detection of GUS activity and sectioning was performed as described in Donnelly et al. (1999). Leaf tissues were cleared in 70% ethanol and 8:2:1 (chloral hydrate:glycerol:water) and mounted in the same solution on microscope slides. For confocal analysis, embryos at different developmental stages were dissected from the seed coat and mounted on coverslips in 5% glycerol, while leaf primordia were dissected from the SAM and mounted onto coverslips in water supplemented with 0.1% Silwet and 25 μg/ml Propidium Iodide. In vitro embryo culture was essentially performed as described (Friml et al., 2003). In brief, excised ovules were placed for 24 hr in the dark at 21°C in 0.5 X MS media containing 2% sucrose and 400 mg/l glutamine with or without 0.1 mM IAA. Embryos were dissected from the ovules and mounted on coverslips in 5% glycerol. Confocal laser scanning microscopy was conducted on a Nikon inverted fluorescence microscope equipped with a Nikon water immersion objective and a Bio-Rad Radiance 2000 confocal head. Images were processed in Adobe Photoshop (Adobe Inc.).

Determination of ABA Levels

The extraction and quantitative analyses, of ABA by GC-EIMS were carried out as described previously (Cheng et al., 2002). [1,2-$^{13}C_2$]-(±)-ABA was used as an internal standard (Asami et al., 1999). Siliques (0.25 g) and 7- and 9-day-old whole seedlings (0.5 g) were used for ABA measurement.

Extraction of Seed Proteins

For seed storage protein analysis 20 wild-type seeds or four emerging vegetative leaves from wild-type and *ML1::FUS3* plantlets were extracted and analyzed by SDS-PAGE as described in Keith et al. (1994).

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